PB87-115127

Immune Function in Adult C57BL/6J Mice Following Exposure to Urethan Pre- or Postnatally

(U.S.) Health Effects Research Lab.
Research Triangle Park, NC

1986

U.S. Department of Commerce
National Technical Information Service
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TECHNICAL REPORT DATA

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1. REPORT NO.	2.	3. RECIP
EPA/600/J-86/174		PB87-115127

4. TITLE AND SUBTITLE
Immune Function in Adult C57bl/6J Mice Following Exposure to Urethan Pre-or Postnatally (Journal Version)

5. REPORT DATE
1986

6. PERFORMING ORGANIZATION CODE

7. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NO.

9. PERFORMING ORGANIZATION NAME AND ADDRESS
PTB/DBD Health Effects Research Laboratory
U.S. Environmental Protection Agency
Northrop Services, Inc., Research Triangle Park, NC

10. PROGRAM ELEMENT NO.

11. CONTRACT/GRANT NO.

12. SPONSORING AGENCY NAME AND ADDRESS
Health Effects Research Laboratory - RTP, NC
Office of Research and Development
U.S. Environmental Protection Agency
Research Triangle Park, NC 27711

13. TYPE OF REPORT AND PERIOD COVERED

14. SPONSORING AGENCY CODE
EPA/600/11

15. SUPPLEMENTARY NOTES
REF: J. Immunopharmacol., 8(2):243-257, June 1986

16. ABSTRACT

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17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS (This Report)	21. NO. OF PAGES
RELEASE TO PUBLIC	UNCLASSIFIED	16
	20. SECURITY CLASS (This page)	22. PRICE
	UNCLASSIFIED	

EPA/600/J-86/174
JOURNAL ARTICLE

JOURNAL OF IMMUNOPHARMACOLOGY, 8(2), 243-257 (1986)

IMMUNE FUNCTION IN ADULT C57BL/6J MICE FOLLOWING EXPOSURE TO URETHAN PRE- OR POSTNATALLY

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ABSTRACT

Administration of urethan (URE or ethyl carbamate) to mice results in the development of a variety of tumors, and, in certain strains of mice, marked suppression of the immune response. Perinatal exposure of mice to URE has been found to result in increased tumor induction compared to exposure of adult animals. In the present study, the effects of perinatal exposure to URE on the development of immunocompetence was investigated. Pregnant mice were injected with total doses of either 0.5 or 1.0 mg URE/g of body weight over days 7-16 of gestation or pups of nontreated dams were administered a total dose of 2.0 mg URE/g of body weight over postpartum days 5-14. Postnatal exposure to URE suppressed NK (natural killer) cell activity but left intact other measured parameters of the host defense system. Prenatal exposure, on the other hand, resulted in elevated leukocyte counts and a trend toward increased spleen and thymus size in offspring of treated mothers. Humoral immune function, as measured by the IgM response to sheep erythrocytes, was suppressed in pups from dams injected with a total of 1.0 mg/g URE. These results indicate that marked differences in immunopharmacologic effects may be observed if chemical exposure occurs at different times during the ontogeny of the immune system.

INTRODUCTION

Urethan (URE) or ethyl carbamate, a known carcinogen which also suppresses cellular replication (1) has been used previously as an antineoplastic agent (2). Use in humans in the United States has been curtailed due to the toxicity and demonstrated carcinogenicity of URE as well as by the advent of more efficacious anticancer drugs. However, as late as 1975, URE was reported to be a constituent in a number of drug

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0163-0571/86/0802-0243$3.50/0

and cosmetic products produced in Japan and intended for humans use (3). In addition, a number of foods and beverages have been identified which contain URE, possibly as a naturally occurring substance and sometimes as the result of processing methods (4).

Leukemia, lung adenomas and other tumor types have been found in laboratory animals exposed to URE while in utero, as neonates by direct injection or via the milk treated dams, or as adults (5). Fetal and newborn mice are more sensitive than adults to tumor induction by URE (6), with the most sensitive period being the last week of gestation (reviewed in 5). Given that carcinogens are often immunosuppressive (7-9), as is the case for URE (10-12), we were interested in the effects of URE on the development of immune function in mice exposed to the compound while in utero or during early postnatal life. Our interest was stimulated by previous reports which indicated that adult C57Bl/6 mice are relatively resistant to URE-mediated immunodepression compared to A/J mice (11) and that the immune system of very young (i.e., 5-17 days old) A/J mice was more sensitive than that of adults of the same strain (10). The purpose of this study was to determine whether the developing immune system of the normally URE-resistant C57BL/6J strain is sensitive to URE. Mice were exposed to URE from day 7-16 of gestation by injecting dams or by direct injection of pups for ten consecutive days, beginning on the fifth postnatal day. At approximately eight weeks of age, lymphoproliferative responses to mitogens and allogeneic cells, natural killer cell activity, delayed hypersensitivity responses and IgM (primary) antibody responses to sheep erythrocytes were evaluated. In addition, body, spleen and thymus weights were measured and organ to body weight ratios calculated.

MATERIALS AND METHODS

Mice: Male and female C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME) and held for at least a 10 day acclimation period before

use. Mice were housed on sterilized wood chips, provided food and water ad libitum, and maintained on a 12 h light/dark cycle at 22 ± 0.5 °C and 50% relative humidity.

Chemical Exposure: Urethan (URE, ethyl carbamate) was obtained from Sigma Chemical Co. (St. Louis, MO) and dissolved in saline for subcutaneous (sc) injection. Dosages employed in this study are known to be tumorigenic when administered to C57Bl/6 mice as a single dose. However, the dosage was divided to investigate the effects of subtumorigenic doses over time and to avoid the hypnotic action of URE at tumorigenic dose levels.

For early postnatal exposure (experiment 1), pregnant females were allowed to deliver and litters were randomly assigned to either treatment or control groups. The amount of URE to be administered was based on the mean weight of all pups within a dose group; the concentration of URE solution was adjusted to contain the appropriate amount in 10 µl. Controls received 10 µl of saline. Mean group weights were determined every 4 days and the concentration of the URE solution adjusted such that 0.2 mg URE/g of body weight per day was given subcutaneously for 10 days beginning on day 5 postpartum.

For in utero exposure (experiments 2 and 3), two virgin females were placed with a single male late in the afternoon and plug-positive females removed the following morning for random assignment to treatment or control groups. URE was administered as sc injections in saline to pregnant females from gestational day 7-17 at rates of either 0.05 or 0.1 mg URE/g of maternal body weight, based on daily maternal body weight.

Hematology: Offspring were sacrificed approximately 8 weeks postpartum. Peripheral blood samples were collected from the retro-orbital sinus of mice lightly anesthetized with ether and immediately diluted in counting fluid (Isoton; Coulter Electronics, Hialeah, FL) for counting erythrocytes and leukocytes, and determination of hematocrit values, using an electronic

particle counter (Coulter Model ZB1). Thin blood smears were prepared and differential leukocyte counts performed (VetPath Labs, Teterboro, NJ).

Lymphoproliferative (LP) Responses: Animals used for hematologic determinations were also used for assesment of LP responses, body weights and selected lymphoid organ weights. Mice were first weighed and bled, then killed by cervical dislocation. Spleens were removed aseptically and weighed, as was the thymus. Single cell suspensions were prepared in complete medium by rupturing the splenic capsule with a sterile syringe plunger; clumps of cells were disbursed by repipeting the cell suspensions through siliconized pasteur pipets. Complete medium consisted of RPMI 1640 containing 25 mM HEPES buffer, 2 mM l-glutamine (Gibco Laboratories), and 50 µg/ml gentamicin (Shering Corp., Union, NJ) supplemented with 5% heat inactivated human AB serum (Irvine Scientific, Santa Ana, CA). Debris were allowed to settle for 10 min at unit gravity and erythrocytes present in the cell suspension were lysed by resuspending pelleted cells in 0.83% ammonium chloride for 3 min. Cells were washed in complete medium and resuspended for hemacytometer cell counts and assessment of viability by trypan blue dye exclusion. LP responses to optimal concentrations of the plant lectins concanavalin A (Con A, 0.2 µg/well, Difco Labs, Detroit, MI) and purified phytohemagglutinin (PHA-P, 0.1 µg/well, Burroughs Wellcome Co., Research Triangle Park, NC) and E. coli lipopolysaccharide (LPS 0111:B4, 1.0 µg/well, Difco Laboratories) were determined in a previously described microculture system (13). Cultures were pulse-labeled for the last 4 h of culture by the addition of 0.5 µCi/well of tritiated thymidine ([^{3}H]TdR, specific activity 6.7 Ci/mmole, New England Nuclear, Boston, MA). Proliferative responses to allogeneic spleen cells (CBA/J) treated with mitomycin C (Sigma, St. Louis, MO) and washed 3X prior to coculture (5×10^5 responder + 1×10^6 stimulator cells) were measured in 96 h microcultures in complete medium supplemented with 5×10^{-5} M 2-mercaptoethanol (Sigma). Cultures were pulsed with 1 µCi [^{3}H]TdR/well 4 hours prior to harvest.

Natural Killer (NK) Cell Assay: Specific release of ^{51}Cr from labeled YAC-1 target cells was measured at three effector-to-target (E:T) cell ratios as previously described (14).

Antibody Response: The plaque forming cell (PFC) assay described by Cunningham (15) was employed to evaluate the humoral immune response to sheep red blood cells (SRBC, Flow Laboratories, McLean, VA). Eight week old offspring were injected intravenously with 0.2 ml of a 5% suspension of washed SRBC four days prior to assay. Mice were killed by cervical dislocation, spleens were removed and cell suspensions prepared in RPMI 1640 containing 2 mM l-glutamine, 25 mM HEPES buffer and 50 µg/ml gentamicin.

Delayed Hypersensitivity Response (DHR): A modification (16) of Lefford's radiometric ear assay (17) was employed to measure DHR responses to keyhole limpet hemocyanin (KLH, CalBiochem, San Diego, CA). Briefly, mice were sensitized to KLH and challenged with KLH in the pinna of one ear; the contralateral (unchallenged) ear was injected with saline only (control). Accumulation of ^{3}H-labeled bone marrow-derived monocytes was measured by cutting a plug of tissue from injection sites, solubilizing the tissue samples (NCS tissue solubilizer, Amersham, Arlington Heights, IL) and counting in a scintillation spectrometer. An index of reactivity was calculated by the formula:

$$\text{DHR Index} = \frac{\text{cpm of KLH ear}}{\text{cpm of saline ear}}$$

Statistical Analysis: All data were subjected to two-way analysis of variance (SAS Institute, Cary NC) to detect dose and sex interactions within an experiment. If a significant interaction ($p<0.01$) was not detected, values for males and females were pooled by dose group and results presented as treatment means; otherwise males and females are presented separately. Significant ($p<0.05$) differences between mean

values for control (saline-injected) and experimental mice were evaluated by Student's t test if there was one treatment and one control group (experiments 1 and 2) and by the Williams (18) test when there were multiple treatment groups (experiment 3). All values are expressed as the mean ± standard error.

RESULTS

Mice administered URE postnatally (experiment 1) or born to dams given 0.05 mg/g/d URE from the seventh to the sixteenth day of pregnancy (experiment 2) had body weights similar to their respective control groups at the time of assay (Table 1). Likewise, body weights and spleen and thymus to body weight ratios were not significantly different in these two experiments with the exception of a relatively small but statistically significant increase ($p<0.05$) in the spleen weight to body weight ratio of URE-treated mice in experiment 2. A significant interaction of treatment and sex was detected in experiment 3 for body weight and thymus to body weight ratios. Other weight parameters, although free of statistically significant interaction of dose and sex effects, are presented separately by sex since ratios were calculated from body weights. Males had reduced body weights and slightly increased thymus weights, resulting in increased thymus to body weight ratios.

Hematologic evaluation revealed no differences between control mice and those exposed to URE for 10 days beginning on day 5 of life (Table 2). Conversely, offspring of dams injected with URE from gestational day 7-16 had elevated ($p<0.05$) white blood cell counts in experiments 2 and 3. The relative distribution of leukocyte types was significantly altered in the second experiment, as treated mice had a smaller percentage of lymphocytes and a larger percentage of neutrophils than offspring of control mice. A statistically significant decrease in numbers of red cells was also detected in treated offspring in experiment 2.

Table 1. Body and Selected Lymphoid Organ Weights Following Urethan (URE) Exposure

Experiment[a]	Daily Dose (N)[b]	Body Weight (g)	Spleen Weight (mg)	Spleen Weight / Body Weight	Thymus Weight (mg)	Thymus Weight / Body Weight
1	Saline (11)	19.0 ± 0.9	75.7 ± 8.0	3.95 ± 0.34	71.8 ± 2.6	3.88 ± 0.25
	0.2mg/g URE (11)	19.7 ± 0.6	71.0 ± 3.6	3.62 ± 0.17	65.9 ± 3.1	3.39 ± 0.20
2	Saline (12)	17.1 ± 0.7	67.1 ± 4.3	3.91 ± 0.16	81.5 ± 2.6	4.86 ± 0.25
	0.05mg/g URE (7)	16.6 ± 0.8	77.4 ± 5.8	4.67 ± 0.25[c]	86.0 ± 3.7	5.26 ± 0.35
3	Saline F (6)	15.5 ± 0.4	60.7 ± 2.5	3.93 ± 0.20	72.5 ± 3.6	4.70 ± 0.27
	0.05mg/g/d,F(6)	15.6 ± 0.5	61.0 ± 7.7	3.88 ± 0.40	69.5 ± 6.9	4.40 ± 0.33
	0.1 mg/g/d,F(6)	14.8 ± 0.3	59.3 ± 4.2	4.01 ± 0.26	74.2 ± 3.0	5.01 ± 0.15
	Saline M (6)	20.5 ± 0.3	72.0 ± 2.3	3.52 ± 0.11	64.2 ± 2.9	3.13 ± 0.11
	0.05mg/g/d,M(6)	17.7 ± 0.5[c]	76.3 ± 8.2	4.27 ± 0.40	80.3 ± 4.6	4.52 ± 0.20[c]
	0.1 mg/g/d,M(6)	17.3 ± 0.4[c]	81.3 ± 4.8	4.69 ± 0.23	77.0 ± 3.6	4.45 ± 0.18[c]

[a] 1 = 10 subcutaneous (sc) injections over days 5-14 after birth, inclusive; 2 and 3 = 10 sc injections over gestational days 7-16, inclusive.
[b] Combined number of females (F) and males (M) unless indicated otherwise.
[c] $p<0.05$.

Table 2. Hematologic Profile of Mice Exposed to Urethan (URE) Pre- or Postnatally

Experiment[a]	Daily Dose (N)[b]	Leukocytes/ mm^3	Percent Lymphocytes	Percent Neutrophils	Percent Monocytes	Erythrocytes/ mm^3 ($\times 10^{-6}$)	Hematocrit (%)
1	Saline (11)	6,163 ± 454	87.9 ± 0.7	8.4 ± 1.1	2.5 ± 0.5	5.92 ± 0.16	29.8 ± 0.9
	0.2mg/g URE (11)	7,587 ± 855	88.5 ± 1.1	8.5 ± 0.8	2.7 ± 0.4	6.31 ± 0.24	32.2 ± 1.4
2	Saline (12)	9,632 ± 1369	87.9 ± 1.0	10.9 ± 1.3	1.2 ± 0.6	7.76 ± 0.17	36.6 ± 0.9
	0.05mg/g URE (7)	20,985 ± 3115[c]	84.3 ± 1.4[c]	14.1 ± 1.1[c]	1.0 ± 0.4	7.03 ± 0.27[c]	34.9 ± 1.1
3	Saline (12)	4,348 ± 552	86.6 ± 1.8	10.2 ± 1.6	2.3 ± 0.5	7.79 ± 0.18	36.4 ± 0.8
	0.05mg/g (12)	3,992 ± 347	91.4 ± 1.5	6.6 ± 1.3	1.6 ± 0.5	7.67 ± 0.18	36.0 ± 0.8
	0.1mg/g (12)	5,655 ± 496[c]	89.3 ± 2.2	8.7 ± 1.9	1.8 ± 0.4	7.50 ± 0.20	35.1 ± 1.0

[a] 1 = 10 subcutaneous (sc) injections over days 5-14 after birth, inclusive; 2 and 3 = 10 sc injections over gestational days 7-16, inclusive.
[b] Combined number of females (F) and males (M) unless indicated otherwise.
[c] $p<0.05$.

Results presented in Table 3 show no alteration in the proliferative response of splenocytes of treated or control mice to mitogens or allogeneic cells in any of the experiments. The values for cpm of incorporated [^{3}H]TdR in the LP response to mitogens in experiment 3 were much lower than historical control values but, as in experiment 2, were similar to control values. Lower levels of NK activity were detected in mice dosed postnatally with URE (experiment 1) at effector to target (E:T) cell ratios of 50: and 100:1 (decreases of 20% and 16%, respectively, $p<0.05$, Table 3). Mice exposed prenatally to URE expressed levels of activity similar to saline injected controls.

Mice treated with URE from day 5-14 of life demonstrated statistically insignificant trends toward diminished antibody production on a per cell and a per spleen basis (Table 4). A significant interaction of dose and sex was observed in numbers of viable cells per spleen in this experiment; treated female offspring had increased splenic cellularity whereas males demonstrated decreased cellularity. Numbers of PFC per million cells and per spleen were decreased by almost half ($p<0.05$) in progeny of dams administered the higher dose of URE in experiment 3. There was no effect on splenic cellularity in experiment 3.

Delayed hypersensitivity responses (DHR) to KLH were similar in saline and URE dose groups in experiments 1 and 3 (data not shown). Mice treated with URE in experiment 2 had DHR index value of 1.73 ± or approximately half that of saline controls (3.12 ± 0.22, $p<0.05$).

DISCUSSION

There were no effects of postnatal URE treatment on body and lymphoid organ weights, hematologic profiles or LP responses to mitogens and alloantigens. There was, however, reduced NK activity in these mice whereas mice exposed *in utero* were not affected. These results are interesting in light of a report by Koo *et al.* (19) describing low numbers of cells bearing

Table 3. Effects of Pre- or Postnatal Exposure to Urethan (URE) on Lymphoproliferative Responses and Natural Killer Cell Activity

Experiment[a]	Daily Dose (N)[b]	Cells Only	[^{3}H]TdR Incorporation-Net CPM (x 10^{-3})[c]				% Specific ^{51}Cr Release[d]		
			Con A	PHA	LPS	MLR	25:1	50:1	100:1
1	Saline (11)	0.19 ± 0.03	25.5 ± 2.6	18.4 ± 1.5	11.3 ± 1.0	24.6 ± 0.6	11.7 ± 0.6	17.2 ± 0.7	21.5 ± 0.6
	0.2mg/g (11)	0.23 ± 0.07	32.3 ± 6.4	17.3 ± 2.5	10.7 ± 1.0	23.5 ± 1.0	10.3 ± 0.8	13.7 ± 0.8[e]	17.7 ± 1.1
2	Saline (12)	1.06 ± 0.13	49.4 ± 6.1	10.0 ± 1.0	17.6 ± 1.9	ND[f]	5.6 ± 0.5	9.4 ± 0.7	10.8 ± 0.7
	0.05mg/g (7)	1.37 ± 0.09[e]	50.9 ± 7.4	7.9 ± 1.4	18.4 ± 1.9	ND	5.4 ± 0.4	7.6 ± 0.4	9.4 ± 0.6
3	Saline (10)	0.22 ± 0.04	3.9 ± 0.6	7.9 ± 0.7	4.3 ± 0.6	18.7 ± 1.9	6.6 ± 0.4	9.5 ± 0.5	13.2 ± 0.6
	0.05mg/g URE (12)	0.24 ± 0.03	5.6 ± 2.2	9.0 ± 1.6	5.6 ± 0.8	17.7 ± 3.6	6.0 ± 0.6	8.7 ± 0.7	10.6 ± 1.1
	0.1 mg/g URE (12)	0.23 ± 0.03	3.3 ± 0.8	6.4 ± 0.8	4.3 ± 0.6	19.1 ± 3.3	7.2 ± 0.9	10.1 ± 1.1	11.9 ± 1.3

[a] 1 = 10 subcutaneous (sc) injections over days 5-14 after birth, inclusive; 2 and 3 = 10 sc injections over gestational days 7-16, inclusive.
[b] Combined number of females (F) and males (M) unless indicated otherwise.
[c] Net cpm = cpm of stimulated cells - cpm of nonstimulated cells; Con A = concanavalin A, PHA = purified phytohemmagglatinin, LPS = *E. coli* lipopolysaccharide, MLK = one-way mixed lymphocyte response to allogeneic splenocytes.
[d] At specified effector to target cell ratio.
[e] $p<0.05$.
[f] Not determined.

Table 4. Primary Antibody Response (PFC) to Sheep Red Blood Cells in Mice Exposed In Utero or Postnatally to Urethan (URE)[a]

Experiment[b]	Daily Dose (N)[c]	Plaque Forming Cells per 10^6 Splenocytes	Plaque Forming Cells per Spleen ($x10^{-4}$)	Cells per Spleen ($x10^{-8}$)
1	Saline (10)	257.0 ± 65.2	5.05 ± 1.09	F(5) 1.59 ± 0.15 M(5) 2.57 ± 0.10
	0.2mg/g URE (10)	162.1 ± 38.1	3.52 ± 0.82	F(4) 2.26 ± 0.08[d] M(6) 2.13 ± 0.03[d]
2-ND[e]				
3	Saline (12)	547.3 ± 58.8	7.61 ± 1.05	1.37 ± 0.07
	0.05mg/g URE (11)	633.9 ± 31.3	9.55 ± 0.64	1.51 ± 0.07
	0.1mg/g URE (6)	299.6 ± 62.3[d]	4.05 ± 0.93[d]	1.33 ± 0.06

[a] Mice were injected intravenously with 0.2 ml of a 5% (v/v) suspension of sheep erythrocytes 4 days prior to assay
[b] 1=10 subcutaneous (sc) injections over days 5-14 after birth inclusive; 3=10 sc injections over gestational days 7-16, inclusive
[c] Combined number of females (F) and males (M) unless indicated otherwise
[d] $p<0.05$
[e] ND = Not determined

the NK-1 surface antigen in C57BL/6 mice prior to day 16 of gestation, but increasing numbers of surface marker-positive cells after birth which peak from 1-3 weeks after birth. It therefore appears that URE administration during very early ontogeny of NK cells has no effect on later development of normal levels of NK activity whereas exposure during the later stages of ontogeny causes a deficiency in NK activity in adults. Furthermore, the effect of URE on NK activity in the C57BL/6 strain may be dependent on exposure of a predominantly immature population of NK cells, as found in animals less than 3 weeks old (19), since NK activity in adult C57BL/6J mice is resistant to URE-mediated suppression (11, Luebke et al., submitted for publication). Similarly, it has been shown that in A/J mice, which are more sensitive to the immunotoxic effects of URE, that suppression of NK activity is greater in mice exposed at 5-17 days of age than in adults

(10). The age-associated differences in host responses may be related to a much lower rate of URE elimination in mice less than 15 days old (reviewed in 5).

Prenatal administration of URE, on the other hand, affected both nonspecific (i.e., hematologic and weight data) and specific indicators of immune system integrity. Numbers of circulating leukocytes were increased in both experiments 2 and 3. These counts may indicate the development of a leukemogenic process, a finding previously reported in this strain of mouse treated with URE (20). We have no evidence of this however, since the relative distribution of leukocytes by cell type was for all practical purposes not affected by URE treatment. A significant dose and sex interaction was seen in thymus to body weight ratios in experiment 3 (Table 1). Lower body weights in treated male mice and a statistically insignificant but apparent mean increase in thymus weights of treated males contributed to this finding. Increased thymus weights have been reported to follow an initial suppression in weight of this organ in treated C57BL mice (20).

Significant suppression of the PFC response to SRBC was observed in experiment 3, both on the basis of PFC/10^6 cells and PFC/spleen. This occurred without a concomitant decrease in splenic cellularity. A study by Parmiani et al. (21) similarly reported that URE exposure causes suppression of the primary immune response to SRBC. In their study, using C3Hf and SWR mice, suppression was induced by treating suckling (10-20 days old) mice with 1 mg URE/g of body weight whereas in the present study, suppression of the response was only observed in mice exposed *in utero*. The lack of significant effects of postnatal treatment on the PFC response is probably the result of the relative resistance of the C57BL strain of mouse to the tumorigenic and immunosuppressive effects of URE (11). There was, however, a trend toward reduced responses by URE-treated mice in the first experiment, suggesting that there may have been an effect

on humoral immunity imposed by chemical exposure that was not expressed strongly at the time of assay. The mechanism of humoral suppression in these studies is unknown at this time and could reside within the helper, accessory, and/or effector cell compartment(s). Given the results of mitogen-driven blast transformation studies of T lymphocytes, there is at this point no evidence for a generalized defect in cell mediated immune functon in these animals. However, defective accessory cell function cannot be ruled out at this point, inasmuch as there is evidence for impaired macrophage function in mice administered URE as adults (12,22).

Our results suggest that in utero exposure to URE may result in either defective or delayed acquisition of primary humoral immune function in the C57BL/6J strain of mice. The pattern of responses in the present study are in contrast to what we observed in C57BL/6J mice exposed to URE as adults; i.e., decreased numbers of circulating WBC's, decreased weights of the spleen and thymus, marked suppression of T cell blastogenic responses to mitogens, without impairment of antigen-evoked production of specific antibody (Luebke, et al., submitted for publication). Our studies, as well as the work of Gorelik and Herberman (10), serve to emphasize the differences in immunopharmacologic effects of chemical exposure of the developing and mature immune systems.

ACKNOWLEDGMENTS

We thank Drs. Anne Tucker and Chris Gordon for helpful comments and suggestions and Ms. Faye Gregory for preparation of the manuscript.

DISCLAIMER

The research described in this article has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the content

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